Lasers in Surgery and Medicine 22:281–287 (1998)

Laser Photostimulation of Collagen Production in Healing Rabbit Achilles Tendons

G. Kesava Reddy, PhD, Lisa Stehno-Bittel, PhD, and Chukuka S. Enwemeka, PhD*

Department of Physical Therapy, University of Kansas Medical Center, Kansas City, Kansas 66160-7601

Background and Objective: **Low energy laser photostimulation at certain wavelengths can enhance tissue repair by releasing growth factors from fibroblasts and stimulate the healing process. This study was designed to evaluate the influence of laser photostimulation on collagen production in experimentally tenotomized and repaired rabbit Achilles tendons.**
Study Design/Materials and Methods: **A total of 24 male New Zealand rabbits, ages 10–12 weeks, were used. Following tenotomy and repair, the surgical hind limbs of the rabbits were immobilized in customized polyurethane casts. The experimental animals were treated with a 632.8 nm He:Ne laser daily at 1.0 J cm^{-2} for 14 days. Control animals were sham treated with the laser head. On the fifth day after repair, the casts were removed to allow the animals to bear weight on the lower extremity. The animals were euthanized on the 15th postoperative day, then, the Achilles tendons were excised, processed and analyzed.**
Results: **Biochemical analyses of the tendons revealed a 26% increase in collagen concentration with laser photostimulation indicating a more rapid healing process in treated tendons compared to controls. Sequential extractions of collagen from regenerating tissues revealed that the laser photostimulated tendons had 32% and 33% greater concentrations of neutral salt soluble collagen and insoluble collagen, respectively, than control tendons suggesting an accelerated production of collagen with laser photostimulation. A significant decrease (9%) in pepsin soluble collagen was observed in laser-treated tendons compared to controls. There were no statistically significant differences recorded in the concentrations of hydroxypyridinium crosslinks and acid soluble collagen between treated and control tendons.**
Conclusion: **This study of laser photostimulation on tendon healing in rabbits suggests that such therapy facilitates collagen production in a manner that enhances tendon healing. Lasers Surg. Med. 22:281–287, 1998.** **© 1998 Wiley-Liss, Inc.**

Key words: collagen synthesis; collagen crosslink; laser therapy; tendon repair; solubility of collagen; tenotomy

INTRODUCTION

The use of low energy laser photostimulation has a wide spectrum of application in medicine and dentistry. During the last decade, attention has been focused on the effects of laser photostimulation on a variety of pathological conditions

Contract grant sponsor: Department of Veteran's Affairs Rehabilitation Research and Development Grant.

*Correspondence to: Dr. Chukuka S. Enwemeka, Department of Physical Therapy Education, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160-7601. E-mail: Enwemeka@kumc.edu

Accepted 26 January 1998

including wounds, musculoskeletal complications, and pain [1–3]. Clinical studies have shown low energy lasers to be effective as analgesics [4] and to accelerate the healing of injured tissue [5–10]. Although the beneficial effects of laser photostimulation are now generally accepted [8–13], the mechanism(s) by which laser light facilitates tissue healing remains poorly understood.

Previous studies suggest that laser photostimulation increases ATP synthesis, promotes nucleic acid production and augments cell division [3,14]. In addition, it has been shown that He:Ne lasers are capable of stimulating matrix collagen production by skin fibroblasts [15,16]. In vivo wound healing studies indicate that laser photostimulation enhances collagen synthesis in the wound area resulting in increased tensile strength [8]. Additionally, elevated procollagen mRNA levels have been reported in cutaneous wounds following treatment with a He:Ne laser [13].

Works from our laboratory indicate that the healing of surgically tenotomized Achilles tendons is considerably improved following He:Ne laser treatment [9,11,12,17,18]. In these studies, the therapeutic effects of laser photostimulation were studied by analyzing morphometric, ultrastructural, and biomechanical evaluations of healing rabbit Achilles tendons. Considering the role of connective tissue collagen in tissue repair mechanisms, an investigation of the effects of laser photostimulation on matrix collagen production was warranted.

The purpose of this study was to determine the possible effect of low energy laser stimulation on objective measures of collagen production in healing rabbit Achilles tendons.

MATERIALS AND METHODS

A total of 24 male New Zealand rabbits, ages 10–12 weeks, were used. The animals were housed individually in standard 30.5 × 71 × 51 cm rabbit cages, maintained 22°C, and fed rabbit chow and water ad libitum.

Tenotomy

On the day of surgery each rabbit was weighed and anesthetized with intramuscular injection of 3 mg/kg body weight Xylazene and 35 mg/kg body weight Ketamine. Subsequently, the skin overlying the right Achilles tendon was shaved, scrubbed and a 2 mg/kg body weight lidocaine local subcutaneous anesthesia was administered at the site of tenotomy. Following anesthesia, the right Achilles tendon of each rabbit was tenotomized and repaired as detailed in previous reports [9,11,12,17,18]. Briefly, a longitudinal incision was made lateral to the visible outline of the tendon. By blunt dissection, the tendon was isolated from adjoining tissue and transected ~1.5 cm above its calcaneal attachment. The severed ends were then approximated and sutured. Following skin closure, the surgical limb was immobilized using a custom designed premolded polyurethane splint. To promote rapid recovery from anesthesia, an injection of 0.2 mg/kg body weight Yohimbine was administered. Subsequently, the rabbits were kept warm in an oxygen chamber and observed until they regained consciousness.

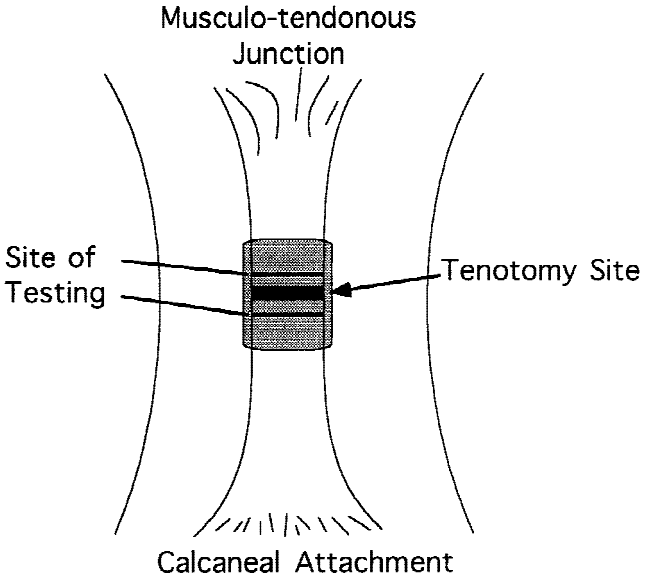

Fig. 1. The schematic representation illustrates the site of tenotomy and the relative location of the laser treatment (shaded area ~5 cm along the new tendon). Further, the figure depicts the region of the neotendon that is tested for the collagen analysis (site of testing).

Treatment

Following tenotomy, an equal number of rabbits were randomly assigned to control and experimental groups. The first group received 1.0 J cm^{-2} He:Ne laser beam (632.8 nm) applied transcutaneously starting on day 1 and continuing for 14 days. The site of application of laser treatment is shown in Figure 1. In addition, to prevent muscle atrophy the gastroesoleus muscle complex of each repaired tendon was stimulated using interrupted galvanic current [19,20] during the first 5 days of immobilization. The treatment protocol

for application of the electrical stimulation was based on previous studies designed to induce maximal mechanical stress without re-rupture [21]. On the fifth postoperative day, immobilization casts were removed from the surgical limbs of the treated and control groups of rabbits to permit free movement of the animals within their cages.

Tendon Excision

Two weeks after surgery, each rabbit was weighed and anesthetized as previously described. Following reopening of the surgical incision, the tendon was separated from the surrounding tissue. Sharp transverse cuts were made below the musculotendinous junction and above the calcaneal insertion of the tendon, then, the neotendon was excised, snap frozen in liquid nitrogen, and stored at –70°C until biochemical analyses were performed.

Measurement of Hydroxyproline

Total collagen was determined by measuring the concentration of hydroxyproline in each tissue specimen as described in our previous report [22]. Briefly, the tissue specimen was homogenized to a fine solution in cold saline, hydrolyzed in alkali, and oxidized with chloramine-T. The chromophore was developed with the addition of Ehrlich's aldehyde and the absorbance of the chromophore was measured at 550 nm. Unknown concentrations of hydroxyproline in each tissue specimen were deduced from a standard calibration curve. The content of total collagen was calculated assuming that 14 percent of the total amino acids of collagen were hydroxyproline.

Determination of Collagen Solubility

The solubility profile of collagen in control and laser treated tendons was examined by sequential extractions in neutral salt buffer, acetate buffer, and acetate buffer containing pepsin as described below.

Neutral salt-soluble collagen (NSC). The tendon samples were cut into small pieces and homogenized in 0.05 M Tris-HCl buffer, pH 7.4 containing 1 M NaCl, 20 mM EDTA and 1 mM PMSF. From the resulting tissue suspension, the NSC was extracted in the same buffer by stirring gently for 24 hr at 4°C. Thereafter, the contents were centrifuged at 15,000g for 30 min at 4°C. The precipitates were suspended twice in neutral salt buffer and recentrifuged. The resulting supernatants were combined and the NSC content was determined by measuring the amount of hydroxyproline.

Acid-soluble collagen (ASC). The residue obtained after the extraction of NSC was suspended in 0.5 M acetic acid, homogenized thoroughly, and the ASC was extracted by stirring gently for 24 hr at 4°C. The contents were centrifuged at 15,000g for 30 min at 4°C, and the precipitate was washed with acetate buffer and recentrifuged. The ASC content in the combined supernatants solution was determined by the hydroxyproline assay.

Pepsin-soluble collagen (PSC). The residue obtained after the extraction of ASC was suspended in 0.5 M acetic acid containing pepsin at 1 mg/ml, and the PSC was extracted by stirring gently for 24 hr at 4°C. Only one digestion was carried out because the purpose was to obtain a measure of collagen stabilization rather than to obtain a maximal yield of solubilized collagen. The contents were centrifuged at 15,000g for 45 min at 4°C, and the resulting precipitate was suspended twice in neutral salt buffer and recentrifuged. The supernatants were combined and analyzed for PSC content using hydroxyproline assay.

Insoluble collagen (ISC). The residue left after the extraction of PSC was homogenized in cold saline, and the hydroxyproline content was determined in the homogenate as a measure of the ISC content.

Measurement of Collagen Crosslinks

The hydroxypyridinium crosslinks were determined as previously reported [23] using a high performance liquid chromatography (HPLC; Shimadzu) with a binary gradient system control module managing a RF-10A spectrofluorometric detector, two pumps, and an auto injector. Briefly, the C18 reverse phase column (Supelco Supelcosil 25 cm × 4.6 mm with 5 mm LC-18 pore size) was equilibrated in 21% acetonitrile in water containing 0.01 M n-heptafluorobutyric acid. Prior to the analysis of collagen crosslinks, samples were hydrolyzed in 6 M HCl for 16 hr at 110°C. The hydrolyzed samples were freeze dried to remove the acid and reconstituted in distilled water. Aliquots of reconstituted sample were then applied to the HPLC column employing an automatic injector. The hydroxypyridinium crosslinks were resolved using a linear gradient from 21% acetonitrile (containing 0.01 M n-heptafluorobutyric acid) to 25% acetonitrile in water. The concentration of hydroxypyridinium crosslinks were measured by determining the area under the peak using pyri-

doxamine as a standard. Since pyridoxamine fluoresces three times greater than the hydroxypyridinium crosslinks, the values obtained from the tendon samples were multiplied by three.

Statistical Analysis

The results were expressed as a mean ± SE. Student's t-test was used to assess biochemical differences between groups of control and treated Achilles tendons. The significance level was predetermined at an alpha level of 0.05.

RESULTS

There was no evidence of infection observed in rabbits following tenotomy and during laser treatment. However, a moderate amount of postoperative swelling of the site of tenotomy was documented in both control and experimental animals. No statistically significant differences were found in the mean body weights of the two groups of animals. No re-rupturing of tendons was noticed in either group of rabbits as well.

The biochemical analysis of collagen indicated that significant differences in the collagen content, collagen solubility profile, and distribution of soluble collagen existed between control and laser-treated tendons. The total collagen for laser-treated tendons was significantly higher compared to the control tendons, 395.15 ± 9.5 μg vs. 314.85 ± 11.1 μg/mg dry tissue (Fig. 2; $P < 0.01$). In contrast to the collagen levels, the hydroxypyridinium crosslinks were not influenced by laser photostimulation. The crosslink density was 0.203 ± 0.006 moles/mole collagen for laser treated and 0.220 ± 0.01 moles/mole collagen for control tendons (Fig. 2; $P > 0.05$). Sequential extractions of collagen from the tissue samples indicated that the proportions of neutral salt-soluble collagen (NSC), acid-soluble collagen (ASC), pepsin-soluble collagen (PSC), and insoluble collagen (ISC) were significantly different between laser-treated and control tendons (Fig. 3). These experiments yielded 12.25 ± 0.36 and 9.3 ± 0.29 μg of NSC, 33.87 ± 1.8 and 35.03 ± 1.7 μg of ASC, 191.28 ± 5.6, and 208.16 ± 4.1 μg of PSC, and 157.75 ± 7.0 and 118.59 ± 5.6 μg of ISC for laser-treated and control tendons respectively. Statistical analysis of the solubility profile revealed a significant increase in NSC and ISC and an appreciable decrease in PSC contents in laser-treated tendons compared to control tendons. However, no statistical differences were observed in the ASC content between laser-treated and control tendons.

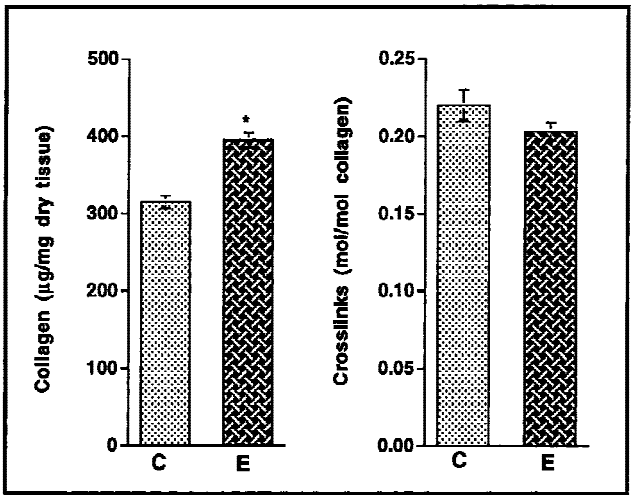

Fig. 2. Influence of laser photostimulation on collagen production and collagen crosslinks in tenotomized and repaired Achilles tendons. C = Control; E = Laser photostimulated tendons. *$P < 0.01$; n = 12. No significant differences were recorded in the amount of collagen crosslinks between control and laser stimulated tendons. $P > 0.05$.

The relative amounts of the distribution of soluble collagens in relation to total tissue collagen are shown in Figure 4. Approximately 3.1% and 2.58% of collagen was extractable in the neutral salt-soluble fraction from laser-treated tendons and control tendons, respectively. Using 0.5M acetic acid, ~8.57% and 9.5% of collagen was extracted from laser-treated tendons and control tendons, respectively. The results of pepsin-digested samples showed that a large amount of collagen was solubilized in both groups of tendons (48.3% laser-treated compared to control tendons, 55.66%). The percent of insoluble collagen was appreciably higher in laser-treated tendons (39.94%) compared to control tendons (32.85%). The sequential extraction and distribution studies of soluble collagens collectively suggest rapid collagen remodeling in the laser-treated tendons compared to the control tendons.

DISCUSSION

Tendon injuries heal slowly and frequently leave scar tissue. Although several therapeutic interventions are available, no method of treatment for moderate or severe tendon injury has been proven to increase the rate, or improve the quality of healing. In recent years, noninvasive laser therapy has gained considerable attention for en-

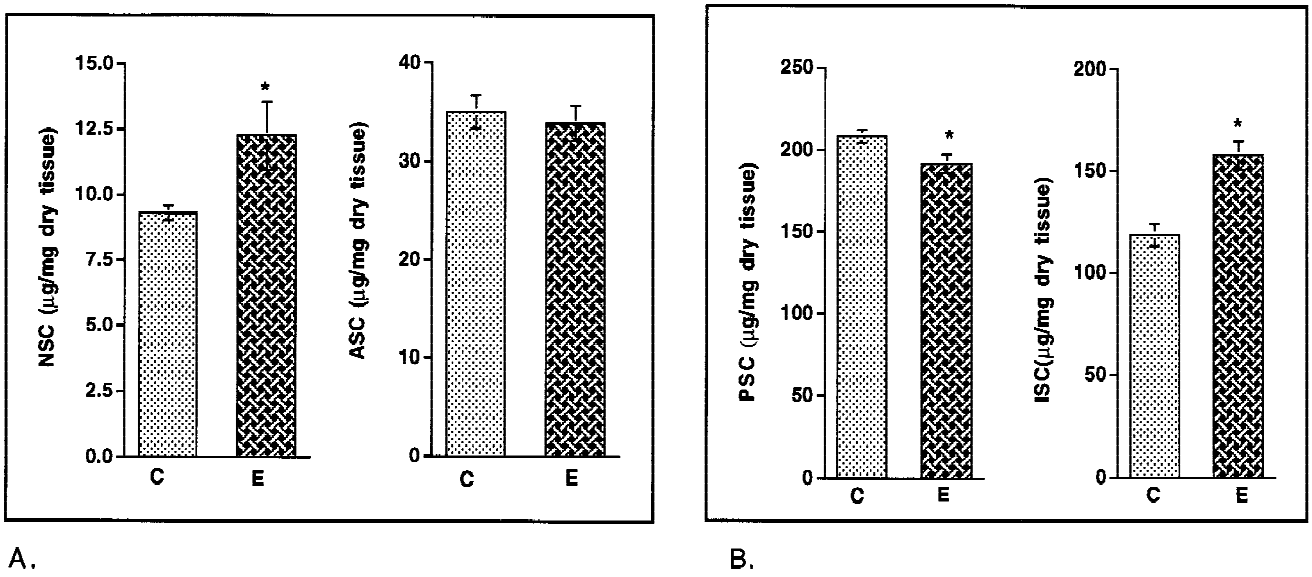

Fig. 3. The solubility of profile collagen in regenerating Achilles tendons. **A.** Comparison of neutral salt-soluble and acid-soluble collagens between control (C) and laser-treated (E) tendons. **B.** Comparison of pepsin-soluble and insoluble collagens between control (C) and laser-treated (E) tendons. Laser-stimulated tendons showed a significant increase in NSC and ISC and a significant decrease in pepsin soluble collagen compared to control. $^*P < 0.01$. No significant changes were documented in the concentration of acid soluble collagen between control and laser treated tendons. $P > 0.01$; n = 12.

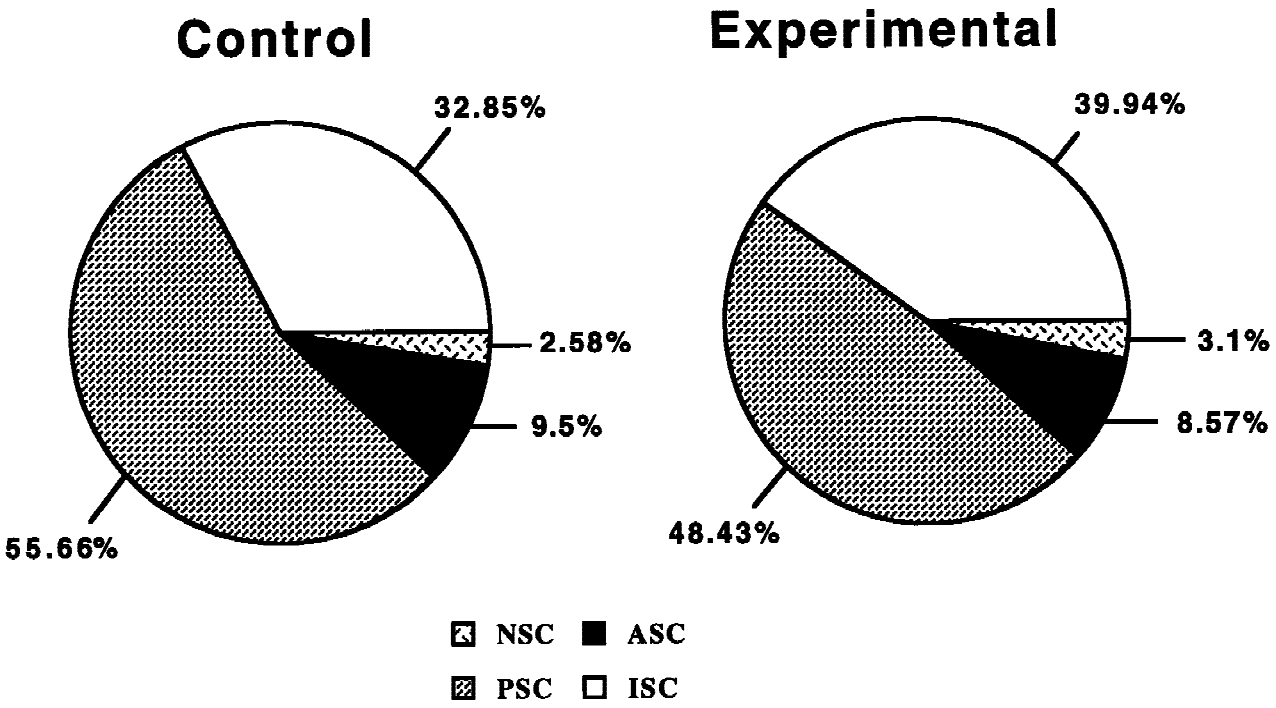

Fig. 4. The distribution of the relative proportions of neutral-soluble, acid-soluble, pepsin-soluble, and insoluble collagen in tenotomized and repaired Achilles tendons. C = Control; E = Laser photostimulated tendons.

hancing wound healing and tissue repair processes [10,24,25]. In our previous studies, we found that He:Ne laser photostimulation accelerates the healing process in repaired Achilles tendons [9,11,12,17,18]. Following treatment with a He:Ne laser, the biomechanical properties improved in healing tendons indicating beneficial effects of laser therapy [9,11,12,17,18]. In this

study, we examined the therapeutic effects of He:Ne laser stimulation on collagen production and its solubility in repaired Achilles tendons.

Collagen production in repaired tendons was evaluated by measuring hydroxyproline concentrations. In addition, we measured and compared the collagen solubility profile and collagen crosslink density of laser treated tendons with control tendons. The results of this study demonstrate that the production of collagen in surgically tenotomized Achilles tendons is modulated by laser photostimulation. The content of total collagen is significantly increased in laser-treated tendons compared to control tendons. In contrast, the treated tendons showed statistically insignificant changes in hydroxypyridinium crosslinks when compared to control tendons. Newly synthesized collagen molecules are known to have immature crosslinks initially, which progressively develop into mature crosslinks. Therefore, it is not surprising that there was no significant differences in hydroxypyridinium crosslinks between treated and control tendons at 14 days.

Our findings demonstrate that the biochemical properties of laser-treated tendons differ widely from those of control tendons. Following sequential extractions, the proportion of neutral salt-soluble collagen and insoluble collagen was significantly higher in laser-treated tendons compared to the control tendons. However, a significant decrease in pepsin-soluble collagen and no change in acid-soluble collagen was observed in laser-treated tendons compared to the control tendons. Pepsin cleaves peptide bonds at the amino terminal of aromatic amino acids such as tryptophan, tyrosine, and phenylalanine. It has been shown that in type I collagen pepsin acts first on the amino terminal end of the molecule, whereas the carboxyl terminal of collagen is more slowly digested [26]. The increased resistance of collagen to pepsin digestion in laser stimulated tendons observed in this study strongly support our previous findings of higher mechanical integrity of Achilles tendons treated with laser [9]. The neutral salt-soluble collagen, a precursor of insoluble collagen, constitutes either the newly synthesized collagen or the degradation product of insoluble collagen [27], suggesting that the turnover rate of collagen was greater in laser-treated tendons than control tendons. Moreover, the solubility of collagen in salts and acids has been correlated with the extent of crosslinking in various connective tissues [28]. The acid-soluble collagen and pepsin-soluble collagen could be considered degradation intermediates of insoluble collagen. Thus our results of collagen solubility clearly suggest that there is rapid synthesis of collagen occurring in the laser-treated tendons. Further, the percentage of distribution of soluble and insoluble collagens indicates that the conversion of soluble to insoluble collagen increased in laser-treated tendons compared to control tendons.

The mechanism by which laser photostimulation facilitates collagen production in regenerating tendons is not clear. It is possible that the stimulation of collagen production in tenotomized tendons by lasers is attributed to alterations in gene regulation or modulation of enzymes that are involved in collagen metabolism. One theory of the mechanism of photostimulation is that the mitochondria are the photoacceptors for light energy [25]. The absorption of energy by the respiratory chain may cause oxidation of NADH, producing changes in the redox status in both mitochondria and cytoplasm. Activation of the electron transport chain results in an increase in the electrical potential across the mitochondrial membrane, an increase in the ATP pool, and finally the activation of nucleic acid synthesis [25]. In vitro studies have shown that laser energy at certain frequencies can modulate cell proliferation and the release of growth factors from fibroblasts [29,30]. Therefore, the positive effects of laser photostimulation on tendon healing may involve the enhancement of growth factors release, which in turn promotes extracellular matrix production and degradation [31,32]. Clearly, further investigation is needed to determine the precise mechanism of action of laser treatment in relation to collagen remodeling and the activities of collagen metabolic enzymes during tendon regeneration.

In summary, the present studies indicate that photostimulation with He:Ne laser leads to enhancement of collagen production in regenerating Achilles tendons. Furthermore, laser photostimulation facilitated the healing process of Achilles tendons by modulating collagen synthesis, thus raising the suggestion that this form of treatment laser photostimulation may be of benefit in postoperation treatment of human tendon ruptures and injuries.

ACKNOWLEDGMENTS

We thank Steve Gum for technical support. The work was supported by the Department of

Veteran's Affairs Rehabilitation Research and Development Grant.

REFERENCES

1. Mester E, Mester AF, Mester A. The biomedical effects of laser application. Lasers Surg Med 1985; 5:31–39.
2. Johannsen F, Hauschild B, Remig L, Johnsen V, Petersen M, Bieler T. Low energy laser therapy in rheumatoid arthritis. Scan J Rheumatol 1994; 23:145–147.
3. Yaakobi T, Maltz L, Oron U. Promotion of bone repair in the cortical bone of the tibia in rats by low energy laser (He-Ne) irradiation. Calc Tiss Intl 1996; 59:297–300.
4. Wedlock P, Shepard RA, Little C, McBurney F. Analgesic effects of cranial laser treatment in two rat nociception models. Physiol Behav 1994; 59:445–448.
5. Abergel RP, Meeker CA, Lam TS, Dwyer RM, Lesavoy MA, Uitto J. Control of connective tissue metabolism by lasers: Recent developments and future prospects. J Am Acad Dermatol 1984; 11:1142–1150.
6. Abergel RP, Dwyer RM, Meeker CA, Lask G, Kelly A, Uitto J. Laser treatment of keloids: A clinical trial and in vitro study with Nd:YAG laser. Lasers Surg Med 1984; 4:291–295.
7. Lam TS, Abergel RP, Castel JC, Dwyer RM, Uitto J. Laser stimulation of collagen synthesis in human skin fibroblast cultures. Laser Life Sci 1986; 1:61–77.
8. Lyons RF, Abergel RP, White RA, Dwyer RM, Castel JC, Uitto J. Biostimulation of wound healing in vivo by a Helium:Neon laser. Ann Plast Surg 1987; 18:47–50.
9. Enwemeka CS. Connective tissue plasticity: Ultrastructural, biomechanical and morphometric effects of physical factors on intact and regenerating tendons. J Orthop Sports Phys Ther 1991; 14:198–212.
10. Romanos GE, Pelekanos S, Strub JR. Effects of Nd:YAG laser on wound healing processes: Clinical and immunohistochemical findings in rat skin. Lasers Surg Med 1995; 16:368–379.
11. Enwemeka CS, Rodriguez O, Gall NG, Walsh NE. Morphometric of collagen fibril population in He:Ne laser photostimulated tendons. J Clin Laser Med Surg 1990; 8:151–156.
12. Enwemeka CS. Laser photostimulation in the United States—A tale of clinical tests, experimental trials, transient triumphs, and intermittent tribulations of potential clinical armamentarium. In Ohshiro T, Calderhead RG, eds. "Progress in Laser Therapy." Chichester, UK: Wiley & Sons, 1991, pp 102–111.
13. Saperia D, Glassberg E, Lyons RF, Abergel RP, Baneux P, Dwyer RM, Castel JC, Uitto J. Demonstration of elevated type I and type III procollagen mRNA levels in cutaneous wounds treated with Helium-Neon laser. Biochem Biophys Res Commun 1986; 138:1123–1128.
14. Passarella S, Casamassima E, Molinari S, Pastore E, Quagliaiello E, Catalano IM, Cingolani A. Increase of proton electrochemical potential and ATP synthesis in rat liver mitochondria irradiated in vitro by helium-neon laser. FEBS Lett 1984; 175:95–99.
15. Abergel RP, Lam TS, Meeker CA, Dwyer RM, Uitto J. Low energy lasers stimulate collagen production in human skin fibroblast cultures. Clin Res 1984; 32:16A.
16. Abergel RP, Lam TS, Castel JC, Meker CA. Biostimulation of procollagen production by low energy lasers in human skin fibroblast cultures. J Invest Dermatol 1984; 82:395–402.
17. Enwemeka, CS. Laser photostimulation. Clin Management 1990; 10:24–29.
18. Enwemeka CS. Ultrastructural morphometry of membrane-bound intracytoplasmic collagen in tendon fibroblasts exposed to He:Ne laser beam. Tissue Cell 1992; 24:511–523.
19. Farkas LG, Herbert MA, James JS. Peritendinous healing after early movement of repaired flexor tendon: Anatomical study. Ann Plast Surg 1980; 5:298–304.
20. Farkas LG, Herbert MA, James JS. Does early movement speed the recovery of function of repaired flexor tendon. Ann Plast Surg 1980; 5:305–308.
21. Venkateswaran V, Basham T, Enwemeka CS. Conference Proceedings of 18th Annual Meeting of American Society of Biomechanics 1994; 109–110.
22. Reddy GK, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem 1996; 29:225–229.
23. Eyre DR, Koob TJ, Van Ness KP. Quantitation of hydroxypyridinium crosslinks in collagen by high-performance liquid chromatography. Anal Biochem 1984; 137: 380–388.
24. Braverman B, McCarthy RJ, Ivankovich AD, Forde DE, Overfield M, Bapka MS. Effect of He:Ne and infrared laser irradiation on wound healing in rabbits. Lasers Surg Med 1989; 9:50–58.
25. Yu W, Naimm JO, Lanzafame RJ. Effects of photostimulation on wound healing in diabetic mice. Lasers Surg Med 1997; 20:56–63.
26. Weiss JB. Enzymatic degradation of collagen. Int Rev Conn Tiss Res 1976; 7:101–157.
27. Jackson DS, Bently JR. Significance of the extractable collagen. J Biophys Biochem Cytol 1960; 7:37–42.
28. Vogel HG. Correlation between tensile strength and collagen content in rat skin. Effect of age and cortisol treatment. Conn Tiss Res 1974; 2:177–182.
29. Yu W, Naim JO, Lanzafame RJ. The effect of laser irradiation on the release of bFGF from 3T3 fibroblasts. Photochem Photobiol 1994; 59:167–170.
30. Yu W, Naim JO, Lanzafame RJ. The effects of photoirradiation on the secretion of TGF-β PDGF and bFGF from fibroblasts in vitro. Lasers Surg Med 1994; (Suppl) 6:8.
31. Chua CC, Geiman DE, Keller GH, Ladda RL. Induction of collagenase secretion in human fibroblasts by growth promoting factors. J Biol Chem 1985; 260:5213–5216.
32. Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH. Transforming growth factor type β: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA 1986; 83:4167–4171.